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(54) Title: DETECTION OF DNA DAMAGING AGENTS (57) Abstract Recombinant DNA molecules comprising a regulatory element that activates gene expression in response to DNA damage operatively linked to a DNA sequence that encodes a light emitting reporter protein, recombinant vectors containing such DNA molecules and cells containing the DNA molecules or recombinant vectors. A method of detecting for the presence of an agent that causes or potentiates DNA damage is also disclosed which involves subjecting the above-described cells to a putative DNA damaging agent and monitoring the expression of the light emitting reporter protein from the cells.		

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DETECTION OF DNA DAMAGING AGENTS

The present invention relates to methods for detecting agents that cause or potentiate DNA damage and to molecules and transformed cells that may be usefully employed in such methods.

DNA damage is induced by a variety of agents such as ultraviolet light, X rays, free radicals, methylating agents and other mutagenic compounds. These agents may cause damage to the DNA that comprises the genetic code of an organism and cause mutations in genes. In microorganisms such mutations may lead to the evolution of new undesirable strains of the microorganism. For instance, antibiotic or herbicide resistant bacteria may arise. In animals these mutations can lead to carcinogenesis or may damage the gametes to give rise to congenital defects in offspring.

These DNA damaging agents may chemically modify the nucleotides that comprise DNA and may also break the phosphodiester bonds that link the nucleotides or disrupt association between bases (T-A or C-G). To counter the effect of these DNA damaging agents cells have evolved a number of mechanisms. For instance the SOS response in *E. coli* is a well characterised cellular response induced by DNA damage in which a series of proteins are expressed, including DNA repair enzymes, which repair the damaged DNA.

There are numerous circumstances when it is important to identify what agents may cause or potentiate DNA damage. It is particularly important to detect agents that cause DNA damage when assessing whether it is safe to expose a person to these agents. For instance a method of detecting these agents may be used as a mutagenesis assay for screening compounds that are candidate food additives, medicaments or cosmetics to assess whether or not the compound of interest induces DNA damage. Alternatively methods of detecting DNA damaging agents may be used to monitor for contamination of water supplies with pollutants that contain mutagenic compounds.

Various methods, such as the Ames Test, for determining the toxicity of an agent are known. More recent developments are disclosed in WO 95/00834 which relates to the use of a light emitting organism (particularly the bacterium *Photobacterium phosphoreum*) for measuring the toxicity of industrial effluents. WO 95/07463 discloses a gene construct formed from DNA encoding for Green Fluorescent Protein and DNA encoding for a regulatory element (such as a promoter induced by heavy metals) which may be used to detect pollution. However these developments do not disclose means of specifically monitoring for the presence of agents that may cause or potentiate DNA damage. Furthermore these known methods are not sensitive enough to detect agents that cause or potentiate DNA damage at low concentrations.

According to a first aspect of the present invention, there is provided a recombinant DNA molecule comprising a regulatory element that activates gene expression in response to DNA damage operatively linked to a DNA sequence that encodes a light emitting reporter protein.

According to a second aspect of the invention, there is provided a recombinant vector comprising a DNA molecule in accordance with the first aspect of the present invention and a DNA vector.

According to a third aspect of the invention, there is provided a cell containing a DNA molecule in accordance with the first aspect of the present invention or a recombinant vector in accordance with the second aspect of the present invention.

According to a fourth aspect of the present invention, there is provided a method of detecting for the presence of an agent that causes or potentiates DNA damage comprising subjecting a cell in accordance with the third aspect of the present

invention to an agent and monitoring the expression of the light emitting reporter protein from the cell.

By "regulatory element" we mean a DNA sequence which regulates the transcription of a gene with which it is associated.

By "operatively linked" we mean that the regulatory element is able to induce the expression of the reporter protein.

By "reporter protein" we mean a protein which when expressed in response to the regulatory element of the DNA molecule of the invention is detectable by means of a suitable assay procedure.

The method of the fourth aspect of the invention is suitable for assessing whether or not an agent may cause DNA damage. It is particularly useful for detecting agents that cause DNA damage when assessing whether it is safe to expose a person to DNA damaging agents. For instance, the method may be used as a mutagenesis assay for screening whether or not known agents, such as candidate foodstuffs, medicaments or cosmetics, induce DNA damage. Alternatively the method of the invention may be used to monitor for contamination of water supplies with pollutants containing DNA damaging agents.

The method of the fourth aspect of the invention may equally be used for assessing whether an agent may potentiate DNA damage. For example, certain agents can cause DNA damage by inhibiting DNA repair (for instance by preventing expression of a repair protein) without directly inflicting DNA damage. These agents are often known as co-mutagens and include agents such as lead.

The regulatory element of the DNA molecule of the first aspect of the invention activates expression of the reporter protein when DNA damage occurs. Such

regulatory elements ideally comprise a promoter sequence which induces RNA polymerase to bind to the DNA molecule and start transcribing the DNA encoding for the reporter protein. The regulatory element may also comprise other functional DNA sequences such as translation initiation sequences for ribosome binding or DNA sequences that bind transcription factors which promote gene expression following DNA damage. Regulatory elements may even code for proteins which act to dislodge inhibitors of transcription from the regulated gene and thereby increase transcription of that gene.

Preferred regulatory elements are DNA sequences that are associated in nature with the regulation of the expression of DNA repair proteins. For instance, the regulatory elements from genes such as RAD2, RAD6, RAD7, RAD18, RAD23, RAD51, RAD54, CDC7, CDC8, CDC9, MAG1, PHR1, DIN1, DDR48, RNR1, RNR2, RNR3 and UB14 from yeast may be used to make molecules according to the first aspect of the invention. There are also regulatory elements associated with inducible excision repair genes in *Neurospora*, inducible recombinational repair genes in *Ustilago* and UV inducible irradiation damage recovery pathway genes in mammalian cells which may be used.

A preferred regulatory element comprises the promoter and 5' regulatory sequences of the RAD54 repair gene. Such a regulatory element may be derived from yeast and particularly *Saccharomyces cerevisiae*. It is most preferred that the regulatory element comprises the promoter and 5' regulatory sequences of the RAD54 repair gene which correspond to the DNA sequence identified as SEQ ID NO 1 or a functional analogue or fragment thereof.

Another preferred regulatory element comprises the promoter and 5' regulatory sequences of the RNR2 gene. The RNR2 gene may be found on chromosome X of *Saccharomyces cerevisiae*. A preferred regulatory element may be derived from between co-ordinates 387100 and 398299 associated with the RNR2

gene on chromosome X as identified in the *Saccharomyces cerevisiae* genome database. The database may be accessed by the World Wide Web at many sites. For example at <http://genome-www.stanford.edu/>.

The DNA sequences that encode a light emitting reporter protein may code for any light emitting protein, however it is preferred that the DNA sequences code for a protein that is fluorescent.

Preferred DNA sequences that encode a light emitting reporter protein code for Green Fluorescent Protein (GFP) and light emitting derivatives thereof. GFP is from the jelly fish *Aequorea victoria* and is able to absorb blue light and re-emits an easily detectable green light and is thus suitable as a reporter protein. GFP may be advantageously used as a reporter protein because its measurement is simple and reagent free and the protein is non-toxic.

Derivatives of GFP include DNA sequences encoding for polypeptide analogues or polypeptide fragments of GFP which are able to emit light. Many of these derivatives absorb and re-emit light at wavelengths different to GFP found endogenously in *Aequorea victoria*. For instance, preferred DNA molecules according to the first aspect of the invention have a DNA sequence that encodes the S65T derivative of GFP (in which serine 65 of GFP is replaced by a threonine). S65T GFP has the advantage that it is brighter than wild-type GFP (when excited at its longest-wavelength peak) and shows only slow photobleaching. Furthermore S65T GFP produces a good quantum yield of fluorescence and matches the output of argon ion lasers used in fluorescence activated cell sorters. Cells according to the third aspect of the invention which contain DNA molecules coding S65T GFP may be used according to the method of the fourth aspect of the invention and are particularly useful when light emission is measured from cell extracts (see below).

Another preferred DNA sequence encodes for a yeast enhanced GFP (YEGFP) such as the GFP derivative described by Cormack *et al.*(1997) (in Microbiology 143 p303 - 311). Such YEGFP has an amino acid sequence which is biased for usage in yeast. Thus YEGFP is particularly suitable for transforming cells according to the third aspect of the invention which are yeast. Furthermore we have found that light emitted from YEGFP in such yeast is even greater than that emitted by S65T derivatives. For example, light output from yeast strain FF18984 (also known as Y486) transformed with a DNA molecule coding YEGFP was double that output from FF18984 transformed with a DNA molecule coding S65T GFP. We have found that YEGFP is particularly suited for use in methods according to the fourth aspect of the invention which involve monitoring light emission from intact cells according to the third aspect of the invention (discussed in more detail below).

DNA molecules coding YEGFP are also useful because YEGFP is less heat sensitive than nascent GFP.

Most preferred DNA molecules according to the first aspect of the invention comprise a RAD54 regulatory element operatively linked to a DNA sequence that encodes a GFP or light emitting derivative thereof.

The DNA molecule may be contained within a suitable DNA vector to form a recombinant vector according to the second aspect of the present invention. The vector may for example be a plasmid, cosmid or phage. Such recombinant vectors are of great utility when replicating the DNA molecule of the first aspect of the invention. Furthermore recombinant vectors are highly useful for transforming cells with the DNA molecule and may also promote expression of the reporter protein.

The recombinant vectors will frequently include one or more selectable markers to enable selection of cells transfected with the DNA vector and, preferably, to enable selection of cells harbouring the recombinant vectors that incorporate the

DNA molecule of the first aspect of the invention. Examples of such selectable markers include genes conferring resistance to kanamycin (or G148) and ampicillin. Selectable markers may include those which restore prototrophy, for example the yeast URA3 gene.

Recombinant vectors may be designed such that the vector will autonomously replicate in the cytosol of the cell. In this case, elements which induce DNA replication may be required in the recombinant vector. A suitable element is derived from the 2 μ plasmid. Such replicating vectors can give rise to multiple copies of the DNA molecule in a transformant and are therefore useful when over-expression (and thereby increased light emission) of the reporter protein is required.

Alternatively the recombinant vector may be designed such that the vector and DNA molecule of the first aspect of the invention integrate into the genome of a cell. Such integration has the advantage of improved stability compared to replicative plasmids. In this case DNA sequences which favour targeted integration (e.g. by homologous recombination) are desirable. For example, incorporation into the recombinant vector of fragments of the HO gene from chromosome IV of *Saccharomyces cerevisiae* favours targeted integration in *Saccharomyces cerevisiae* or cell-lines derived therefrom. It is preferred that the fragment of the HO gene has the sequence identified as SEQ ID No 5 or is a derivative thereof. It is also possible to insert multiple copies of integrating recombinant vectors into the genome. This will allow enable greater expression and increase the signal output further. For instance, the vectors may be targeted to chromosome XII using sequences from the ribosomal DNA array.

Preferably recombinant vectors may be formed from PFA vectors or derivatives thereof which are known to the art (see Wach *et al.* (1994) Yeast 10 p1793-1808).

Preferred DNA molecules according to the first aspect of the invention have a DNA sequence that encodes for a GFP or light emitting derivative thereof that is derived from these PFA vectors.

Preferred recombinant vectors are PFA KANMX3GFP-RAD54, pWDH443 and pWDH444 which are described in detail in the Example. The preferred DNA molecules of the first aspect of the present invention which comprise a RAD54 regulatory element operatively linked to a DNA sequence that codes for a GFP or light emitting derivative thereof may be derived from these most preferred recombinant vectors PFA KANMX3GFP-RAD54, pWDH443 or pWDH444.

Recombinant vector PFA KANMX3GFP-RAD54 comprises the vector of sequence listing SEQ ID NO 4 with the regulatory element of sequence listing SEQ ID NO 1 or a functional analogue or fragment thereof inserted between the PacI and BamHI restriction enzyme sites of the vector of sequence listing SEQ ID NO 4.

Preferred recombinant vector pWDH443 comprises PFA KANMX3GFP-RAD54 with a fragment of the HO gene corresponding to SEQ ID NO 5 inserted at the unique BamHI site of the KANMX3GFP-RAD54. pWDH443 comprises DNA coding for S65T GFP and is capable of integrating into the yeast genome.

Preferred recombinant vector pWDH444 comprises a fragment of the 2 μ plasmid ligated with the large fragment generated from the BamHI and PmeI digestion of pWDH443. The fragment of the 2 μ plasmid may correspond precisely to the large HindIII/BamHI fragment released from the plasmid pRDK249, described in the Journal Biological Chemistry, Volume 266, p14049, Figure 1 (1991) in the article by Johnson, A.W. and Kolodner, R.D. The DNA from the 2 μ plasmid in pWDH444 allows this recombinant vector to autonomously replicate in the cytosol of a host cell and thereby allows the recombinant vector to be present in high copy numbers (which

can be advantageous when over-expression of GFP and increased light emitting capability is desired).

Other preferred recombinant vectors are yEGFP-443 and yEGFP-444 (see Figs 11 and 12).

yEGFP-443 is derived from pWDH443 and comprises the large fragment of pWDH443 generated by PacI and AscI digestion with the PacI and AscI digestion product of SEQ ID NO 6 (which encodes a YEGFP) ligated into the said large fragment. yEGFP-443 comprises DNA coding for YEGFP and is capable of integrating into the yeast genome.

yEGFP-444 is derived from pWDH444 and comprises the large fragment of pWDH444 generated by PacI and AscI digestion with the PacI and AscI digestion product of SEQ ID NO 6 (which encodes a YEGFP) ligated into the said large fragment. The DNA from the 2 μ plasmid in yEGFP-444 allows this recombinant vector to autonomously replicate in the cytosol of a host cell and thereby allows the recombinant vector to be present in high copy numbers (which can be advantageous when over-expression of GFP and increased light emitting capability is desired).

According to the third aspect of the invention the DNA molecule is incorporated within a cell. Such host cells may be prokaryotic or eukaryotic. Suitable host cells include bacteria, plant, yeasts, insect and mammalian cells. Preferred host cells are yeast cells such as *Saccharomyces cerevisiae*. Yeast are preferred because they can be easily manipulated like bacteria but are eukaryotic and therefore have DNA repair systems that are more closely related to humans than those of bacteria. Thus the use of such yeast in the method of the invention represents an improved method for detecting DNA damage relative to the Ames test. The Ames test uses bacteria (strains of *Salmonella typhimurium*) which when exposed to a putative DNA damaging agent may result in a genotoxicity result (positive or negative) which,

because of the differences between prokaryotes and eukaryotes, would not necessarily be representative of the effects of such agents in eukaryotes such as humans.

Another benefit of using yeast cells as a host is that DNA repair systems are inducible in yeast unlike in humans where the repair systems are largely constitutive.

Preferred yeast cells include:

- (i) Y485 in haploid form;
- (ii) Y486 (also known as FF18984) in haploid form
- (iii) Y485/486 in diploid form;
- (iv) FY73
- (v) YLR030w α ; and
- (vi) Y300.

These strains may all be found in national yeast strain collections.

The type of yeast strain used can influence the DNA damage response and we have found that light emission can vary greatly depending upon the yeast strain used. In this respect we have found that (i), (ii) and (iii) above are particularly useful strains for use according to the method of the invention.

Host cells used for expression of the protein encoded by the DNA molecule of the invention are ideally stably transformed, although the use of unstably transformed (transient) cells is not precluded.

Transformed cells according to the third aspect of the invention may be formed according to following procedures. PFA vectors may be used as suitable starting material from which DNA molecules of the first aspect of the invention and recombinant vectors of the second aspect of the invention may be formed. The known PFA vectors contain, or may be manipulated to contain, DNA encoding for GFP and

derivatives thereof. Such vectors may be manipulated, by known molecular biology techniques, to insert a suitable regulatory element adjacent to the GFP coding sequence thus forming a DNA molecule of the first aspect of the invention which is contained within a recombinant vector according to the second aspect of the invention. For instance, pWDH443, pWDH444, yEGFP-443 and yEGFP-444 may be derived from such PFA vectors. The DNA molecule may be excised from the recombinant vector, or more preferably the DNA molecule contained within the recombinant vector may be used to transform a cell and thereby form a cell according to the third aspect of the invention. The cell is ideally a yeast cell (for instance one of the abovedescribed strains). Such transformed cells may be used according to the method of the fourth aspect of the invention to assess whether or not agents induce or potentiate DNA damage. GFP expression is induced in response to DNA damage and the light emitted by GFP may be easily measured using a fluorimeter as an index of the DNA damage caused. For instance, the light emitted by GFP at 511nm (after excitation between 475 and 495nm - e.g. 488nm) in response to DNA damage, may be evaluated either in a suspension of a defined number of whole cells or from a defined amount of material released from cells following breakage.

Many known methods of detecting DNA damage (including the Ames Test and related methods) assay lasting DNA damage, as an endpoint, either in the form of misrepaired DNA (mutations and recombinations) or unrepaired damage in the form of fragmented DNA. However most DNA damage is repaired before such an endpoint can be measured and lasting DNA damage only occurs if the conditions are so severe that the repair mechanisms have been saturated. Preferred methods of the fourth aspect of the present invention are much more sensitive than these known methods because they detect repair activity (which we have found to be detectable when actual DNA damage is undetectable) which prevents the above mentioned endpoint being reached. Therefore the method of the fourth aspect of the invention may be used to detect for the presence of DNA damaging agents or DNA damage potentiating agents at concentrations below the threshold for which actual DNA damage may be detected.

The method of the fourth aspect of the invention is particularly useful for detecting agents that induce DNA damage at low concentrations. The methods may be used to screen compounds, such as candidate medicaments, food additives or cosmetics, to assess whether it is safe to expose a living organism, particularly people, to such compounds.

Alternatively the methods of the fourth aspect of the invention may be employed to detect whether or not water supplies are contaminated by DNA damaging agents or agents that potentiate DNA damage. For instance, the methods may be used to monitor industrial effluents for the presence of pollutants that may lead to increased DNA damage in people or other organisms exposed to the pollution.

When the methods are used to detect whether or not water supplies are contaminated, the cells according to the third aspect of the invention are ideally unicellular organisms such as bacteria, algae, protoza and particularly yeast.

The expression of light emitting reporter protein may be monitored according to the method of the invention from cell extracts (in which case cells transformed with any of the abovedescribed recombinant DNA molecules and/or recombinant vectors may be used) or from samples containing intact, whole cells (in which case yEGFP-443 and yEGFP-444 transformed yeast cells are preferably used).

There are several advantages associated with the use of whole cells. As there is no requirement to break open cells, the number of treatment steps is reduced. The production of extracts requires cell-harvesting, washing, breakage with glass beads and centrifugation to clear the extract. The reduction in treatment steps also reduces the risk of errors arising in handling and makes the method much faster. Furthermore cell density and light emission can be made simultaneously giving greater sensitivity. Therefore the method of the invention is preferably performed by growing cells

transformed with a recombinant vector according to the second aspect of the invention (such as yEGFP-4443 or yEGFP-444), incubating the cells with the agent which putatively causes DNA damage for a predetermined time and monitoring the expression of the light emitting reporter protein directly from a sample of the cells.

When whole cells are used they are preferably contained in low fluorescence growth medium. This can obviate the need to wash the cells before measurements are made and therefore reduce the number of steps in the method further. For instance, preferred yeast according to the third aspect of the invention may be grown in F1 medium (described in Walmsley *et al.* (1983) Mol. Gen. Genet. 192 p361-365).

According to a preferred embodiment of the method of the invention FF18984 cells may be transformed with yEGFP-444 and grown in F1 medium. A putative DNA damaging agent (e.g. a food additive or potential medicament or an agent contained within a water sample or effluent sample) may then be added to the F1 medium containing the cells. The cells are then allowed to grow for a defined period of time after which a sample of the cells is removed and fluorescence measured therefrom. This measurement may be effected by estimating the cell concentration and fluorescence in the sample using nephelometry (light scattering). For example, cells can be illuminated at 600nm and the scattered light (at 600nm) estimated at 90 degrees to the incident beam. The light emitted by GFP can be measured by excitation at 495nm and fluorescent light emitted at 518nm measured at 90 degrees to the incident beam. Both measurements may be made in a single cuvette. A normalised GFP light emission is calculated by dividing the GFP fluorescence value by the whole cell light scattering value (at 600nm). This embodiment of the invention has the advantage that it may be easily carried out with a minimum of steps (i.e an incubation period followed by direct fluorescence measurement).

The method of the invention should ideally employ sensitive fluorimeters and reduce light scattering in order that light emission can be accurately measured from

the reporter protein. We have found that sensitivity can be improved by using a 495nm filter which is introduced between the sample chamber and the emission-detector of the fluorimeter. Such a filter further reduces the impact of light scattering and improves the sensitivity of the method when samples containing whole cells are used.

The present invention will now be described, by way of examples, with reference to the accompanying drawings in which:

Figure 1 is a schematic representation of DNA coding for the RAD54 promoter showing where the PCR primers used in Example 1 bind to the DNA;

Figure 2 is a schematic representation of DNA coding for the RAD54 promoter prepared by PCR amplification as described in 1.1 of Example 1;

Figure 3 is a schematic representation of the PFA KANMX3GFP construct used in Example 1 in the preparation of DNA molecules of the invention;

Figure 4 is a schematic representation of the PFA KANMX3GFP-RAD54 recombinant vector of Example 1 which incorporates a DNA molecule of the invention;

Figure 5 is a schematic representation of the pWDH443 recombinant vector of Example 1 which incorporates a DNA molecule of the invention;

Figure 6 is a schematic representation of the pWDH444 recombinant vector of Example 1 which incorporates a DNA molecule of the invention;

Figure 7 is a graph illustrating an increase in fluorescence occurs in cells according to the third aspect of the invention when the cells are exposed to a DNA damaging agent;

Figure 8 is a graph illustrating the time course of the induction of brightness by a DNA damaging agent for a cell transformed with pWDH444;

Figure 9 is a graph illustrating the time course of the induction of brightness by a DNA damaging agent for a cell transformed with pWDH443;

Figure 10 is a graph illustrating the effect of differing concentrations of a DNA damaging agent on cell extract brightness by a DNA damaging agent for extracts from a cell transformed with pWDH444;

Figure 11 is a schematic representation of the yEGFP-443 recombinant vector of Example 3 which incorporates a DNA molecule of the invention;

Figure 12 is a schematic representation of the yEGFP-444 recombinant vector of Example 2 which incorporates a DNA molecule of the invention; and

Figure 13 is a graph illustrating emission spectra of yeast cells untransformed and transformed cells with yEGFP-444 +/- 0.01% MMS.

EXAMPLE 1

1.1 METHODS

1.1.1 Preparation of DNA coding for the RAD54 promoter.

A DNA fragment was prepared which contained the promoter region of the RAD54 gene and is schematically represented in Fig. 2.

The RAD54 gene is on Chromosome VII of the yeast *Saccharomyces cerevisiae*. The sequence of the entire genome of *Saccharomyces cerevisiae* is available on public databases. For instance the *Saccharomyces cerevisiae* genome database may be accessed by the World Wide Webb at many sites such as <http://genome-www.stanford.edu/>. The relevant region of Chromosome VII which contains the RAD54 gene and regulatory element of interest spans bases 193710-198141.

The DNA fragment containing the promoter region of the RAD54 gene was prepared by Polymerase Chain Reaction (PCR) amplification of the upstream non-coding region of RAD54 between the RAD54 start codon (ATG) and next gene (SUT1). This region spans 1729 base pairs and contains all sequences known to be involved in RAD54 regulation (i.e. the regulatory element). This region is encoded by SEQ ID NO 1.

The PCR amplification was carried out using two PCR primers called BAM54 and 54PAC which are identified as sequence listings SEQ ID NO 2 and SEQ ID NO 3 respectively.

BAM54 comprises the following 31 bases:

5' cctccggatccgacatacgaatgacctaag 3'

BAM54 was designed to contain the bases *gga**tcc* to ensure that the PCR product contained a BamH1 restriction site and the bases **gacatacga***tga***cctcaatg** which correspond to the first 20 bases after the SUT1 gene in order that BAM54 may bind to the desired DNA fragment. The 5' bases *cctcc* were added to ensure BamH1 could cut at the introduced site.

54PAC comprises the following 44 bases:

5' *cctccg****ttaatta****cat****cagttataagg****aa****tatatat****ggtacc* 3'

54PAC was designed to contain the bases *ttaattaa* to ensure that the PCR product contained a PacI restriction site, the bases **cagttataagg***aa****tatatat****ggtacc* correspond to the first 27 upstream bases of the RAD54 gene in order that 54PAC may bind to the desired DNA fragment and the 5' bases *cctccg* were added to ensure PacI could cut at the introduced site. The primer 54PAC forms the antisense strand of the amplified fragment and therefore the bases in 54PAC that ultimately code for a reporter protein of a DNA molecule according to the first aspect of the invention are "antisense codons" such that the bases *cat* were introduced to add the amino acid methionine which acts as a start codon in a recombinant vector of the second aspect of the invention and the bases *cctccg****ttaatta****cat* are also "antisense" coding sequence.

Figure 1 shows a schematic representation of the 5' and 3' portions of the DNA fragment (1) which was PCR amplified and illustrates where primers BAM54 (2) and 54PAC (3) bind to the fragment. Fig 1 further shows the BamH1 restriction site (4) and the PacI site (5) which were introduced into the PCR amplified fragment (1). The transcription start, the codons for the first 5 amino acids of a protein expressed from the RAD54 promoter and the direction of transcription are also shown.

Following PCR amplification the DNA coding for the RAD54 promoter was inserted into a PFA vector to form a recombinant vector of the invention (see 1.3).

1.1.2 The PFA KANMX3GFP construct.

A PFA vector was used in the construction of recombinant vectors according to the invention. The PFA plasmid series has been previously published (Wach *et al.* (1994) Yeast 10 p1793-1808).

A plasmid PFA KANMX3GFP was derived from the known plasmid PFA KANMX3 (shown in Figure 1B, page 1797 of Wach *et al.*) In the plasmid used by the inventors the lacz sequences of PFA KANMX3 were replaced by GFP sequences to form PFA KANMX3GFP. The sequence of PFA KANMX3GFP is attached as SEQ ID NO 4. (see Fig. 3)

PFA KANMX3GFP contains a DNA sequence coding for the S65T derivative of GFP which has a PacI site just after the transcription start codon of the GFP gene. S65T was inserted into the vector as a PacI/AscI fragment. PFA KANMX3GFP also contains DNA (2689bp) encoding for a gene capable of conferring kanamycin resistance.

1.1.3 The KANMX3GFP-RAD54 construct (see Fig. 4)

The DNA coding for the RAD54 promoter (see 1.1) and PFA KANMX3GFP (see 1.2) were treated with the restriction enzymes BamHI and Pac I. The KANMX3GFP-RAD54 construct was then made by uniquely ligating the DNA coding for the RAD54 promoter (with BamHI and Pac I sticky ends) into the linearised PFA KANMX3GFP between the unique BamHI and Pac I site adjacent to the S65T coding sequence. To ease cloning PFA KANMX3GFP was also treated with SmaI. The SmaI site in PFA KANMX3GFP lies between BamHI and PacI, therefore the SmaI treatment prevented recircularisation of the vector. The PacI site is "in-frame" such that the S65T gene may be transcribed.

1.1.4 The pWDH443 construct

The KANMX3GFP-RAD54 construct was cut at the unique BamH1 site, then treated with DNA polymerase (pol1) and calf intestinal phosphatase to produce blunt and un-self ligatable ends. A Dra1 fragment (see SEQ ID NO 5) of the HO gene was then ligated between the blunt ends of the treated KANMX31-RAD54 construct to form the pWDH443 construct.

The HO gene is found on Chromosome IV of the yeast chromosome and sequences are held in the *Saccharomyces cerevisiae* genome database, which is in the public domain. The database may be accessed by the World Wide Web at many sites. For example at <http://genome-www.stanford.edu/>

The Dra1 fragment of the HO gene contains a BamH1 site at bases 711 - 716 of SEQ ID NO 5 and was employed to promote integration of the construct into the yeast genome by the known technique of homologous integration.

The pWDH443 construct can be cut with BamH1 and used to transform yeast (Y485/486) with G418 selection (a derivative of kanamycin). Transformation was done using the known Lithium acetate procedure (see http://www.umanitoba.ca/faculties/medicine/human_genetics/gietz/Trafo.html).

The pWDH443 construct integrates into the yeast genome at the HO locus. pWDH443 is a preferred recombinant vector according to the second aspect of the invention and cells transformed with pWDH443 are preferred cells according to the third aspect of the invention.

1.1.5 The pWDH444 construct (see Fig 6).

The pWDH444 construct was then developed (by manipulation of pWDH443) such that the construct was capable of replication inside a yeast host to be present as

multicopies. To do this a DNA fragment (blunt at one end and BamH1 cut at the other) containing sequences encoding for cis-acting replication factors from the naturally occurring yeast 2 μ plasmid, yeast URA3 gene (uracil prototrophy) and bacterial plasmid pBR322 sequences conferring replicative ability (ORI) and ampicillin resistance (AMPr) to the construct (when maintained in *E. coli*) were ligated into pWDH443 cut with BamH1 and PmcI. This cut removed a fragment of pWDH443 which contained a NotI site. The ligation mix was cut with NotI prior to transformation to prevent recircularisation of pWDH443. The BamH1 site within the HO fragment (see above) and the HO fragment is therefore truncated in the pWDH444.

The 2 μ plasmid fragment contained sequences corresponding to the yeast 2 μ origin, URA3 gene, pBR322 ORI sequence and Ampicillin resistance are found as a contiguous fragment in several plasmids. (For instance the shuttle vector named Yep420.) The 2 μ plasmid fragment used by the inventors corresponds precisely to the large HindIII/BamH1 fragment released from the plasmid pRDK249, described in the Journal Biological Chemistry, Volume 266, p14049, Figure 1 (1991) in the article by Johnson, A.W. and Kolodner, R.D. Specifically, the plasmid was first cut with HindIII, then treated with DNA polymerase 1 to produce a blunt end. The plasmid was then treated with BamH1 producing a fragment blunt at one end and with a BamH1 end at the other. This fragment was purified by gel electrophoresis.

The pWDH444 construct was used to transform yeast (as described for pWDH443) selecting for either uracil prototrophy (URA3) or G418 (kanamycin) resistance and was capable of autonomous replication with the yeast. pWDH444 is a preferred recombinant vector according to the second aspect of the invention and cells transformed with pWDH444 are preferred cells according to the third aspect of the invention.

1.2. FLUORESCENCE DETERMINATION EXPERIMENTS.

Light emitted by GFP at 511nm (after excitation at 488nm) from yeast cells transformed with pWDH443 and pWDH444 and expressing GFP was measured in a fluorimeter in the absence (controls) or presence of compounds known to induce DNA damage such as Methyl Methane Sulphonate (MMS). Samples were assayed as whole cells or as cell extracts.

1.2.1. Strains and Growth Conditions.

Yeast strains Y485 and Y486 in haploid form or Y485/486 in diploid form were in this Example (see above).

The media YP plus 2% glucose (YPD) and synthetic media plus 2% glucose (SD) were prepared as described in Kaiser *et al.* (1994) (Methods in Yeast Genetics. Cold Spring Harbor Laboratory Course Manual, NY: Cold Spring Harbor Press, Cold Spring Harbor Laboratory, NY,NY.).

1.2.2. Fluorescence Assays.

A stationary phase culture of cells grown in SD medium was used as the inoculum source. Aliquots (30µl) of cells were inoculated into 3ml of SD in 15ml test tubes. Half of the tubes were then supplemented with 0.01% MMS (the remaining tubes left as controls). The tubes were incubated at 25°C for 16h on an orbital shaker at 120rpm, then transferred to an ice-box whilst maintaining agitation. The cells were then transferred to 1.5ml eppendorf tubes, harvested by centrifugation (10s), washed twice in sterile distilled water, and resuspended in 250µl extraction buffer.

For whole cell fluorescent measurements, the washed cells were transferred directly to cuvettes containing 2.75ml sterile distilled water.

For cell extract studies, 100µl of 400-600nm diameter glass beads were added to the tubes of cells which were then placed in a BIO 101 Fastprep FP120 to

mechanically disrupt the cells. Following centrifugation for 30s, the supernatant was transferred to a clean tube. The pellet of beads was washed in a further 250µl of extraction buffer and this was added to the supernatant from the previous extraction. 200µl of the pooled extract in 300µl of water was then transferred to a cuvette.

Fluorescence measurements were performed with a Perkin Elmer LS50 Fluorescence Spectrometer. The excitation and emission wavelengths were set to 488nm and 511nm respectively, with a slit width of 10nm. To correct for variations in cell number/protein extraction efficiency, light absorption was recorded for each cuvette at either 600nm (for whole cells) or 280nm (for extracts). The fluorescence values obtained from the fluorometer were then divided by the absorption readings to give the "brightness value", an arbitrary unit independent of sample concentration. Other data handling details are dealt with either in the text or in the

1.2.3 Results

For pWDH443 transformed yeast, both mating types (Y485 and Y486) and the diploid (Y485/486) showed statistically significant increases in fluorescence when exposed to 0.002% and 0.01% MMS.

As shown in Fig 7., pWDH443 transformed yeast (Y485, Y486 and diploid [Y485/486]) also showed an increase in brightness in the presence of the DNA damaging agent MMS (Y485i, Y486i and Diploidi) relative to untransformed cells also exposed to MMS (Y485, Y486 and Diploid).

Fluorescence began to increase one hour after induction with 0.05% MMS. Fig. 8 and Fig. 9 show time courses of the induction of brightness by MMS in the haploid yeast strain Y486 transformed with pWDH444 (Y486p + mms) or pWDH443 (Y486i + mms) respectively. The effect of MMS on untransformed cells (Y486 + mms) and brightness in untreated cells (Y486, Y486p and Y486i) is also shown.

Fig. 10 illustrates that 0.02% and 0.05% MMS causes an increase in fluorescence in the haploid yeast strain Y486 that has been transfected with pWDH444 (Y86p) but has no significant effect in untransformed cells (Y486). SDS was included as a control to abolish fluorescence from GFP.

Protein extracts from untransformed yeast cells and yeast cells transformed with both pWDH443 and pWDH444 were used in a Western blot analysis using anti-GFP antibody. GFP was not detected in untransformed cells and was only detected at low levels in uninduced transformed cells. However when yeast cells transformed with pWDH443 or pWDH444 were induced with MMS there was a considerable increase in GFP antibody binding. This increase was proportional to the measured fluorescence and therefore confirmed that the increase in fluorescence was due to DNA damage inducing the RAD54 promoter and subsequently causing GFP expression.

These data illustrate that yeast transformed with pWDH443 and pWDH444 emit increased fluorescence (at 511nm) in the presence of a DNA damaging agent. Accordingly pWDH443 and pWDH444 are highly preferred recombinant vectors according to the second aspect of the present invention and yeast transformed with pWDH443 and pWDH444 are highly preferred cells according to the third aspect of the invention. These transformed yeast cells are highly suited to be used according to the method of the fourth aspect of the invention although it will be appreciated that alternative recombinant vectors may be constructed according to the second aspect of the present invention and cells transformed with such alternative recombinant vectors will fall within the scope of the present invention.

Cells transformed with pWDH443 and pWDH444 may be used in a method according to the fourth aspect of the present invention as a means of screening compounds, such as candidate medicaments, food additives or cosmetics, to assess whether it is safe to expose a living organism, particularly people, to such compounds

or the cells may be used to detect whether water supplies are contaminated by DNA damaging agents or agents that potentiate DNA damage.

EXAMPLE 2

Recombinant vector yEGFP-444 was formed and used to transform yeast cells which expressed YEGFP in response to a DNA damaging agent (MMS).

2.1. METHODS

2.1.1. Strains and Growth Conditions.

Yeast strain used in this Example:

- (i) FF18984 (*MAT α leu2-3,112 ura3-52 lys2-1 his7-1*)
- (ii) YLR030w α (*MAT α his3 Δ 200 ura3-52*)

The media used was the same as described in 1.2.1.

2.1.2 DNA constructs

The modular design of pWDH444 (see Example 1) allowed simple replacement of the S65T derivative of GFP with the YEGFP gene (Cormack et al, 1997 *supra*) to form preferred recombinant vector yEGFP-444 (see Figs 12).

YEGFP was PCR amplified using primers that added flanking PacI and AscI restriction enzyme sites to yield the PCR product of SEQ ID NO. 6. This PCR product was then cut with PacI and AscI.

The S65T GFP gene was released from pWDH444 by cutting with PacI and AscI, and gel electrophoresis was used to purify the remaining vector fragment (i.e. the large fragment).

The larger PacI and AscI fragment of the PCR product was then ligated into the large fragment of pWDH444 to form yEGFP-444.

2.2 FLUORESCENCE DETERMINATION EXPERIMENTS.

2.2.1 Fluorescence Assays.

The assessment of GFP fluorescence in whole cells was performed essentially as described in 1.2 (above).

2.2.2 Results

Light output from cells transformed with plasmid yEGFP-444 was even greater than from those transformed with pWDH443 or pWDH444. In fact, Table 1 illustrates that light output more than doubled from FF18984 transformed with yEGFP-444 relative to FF18984 transformed with pWDH444.

Table 1: Comparison of fluorescence output from yEGFP-444 and pWDH444.

Recombinant Vector	Brightness (BU) + MMS	Brightness (BU) - MMS
yEGFP-444	100	45
	97	41
	93	47
	average = 96.7	average = 44.3
pWDH444	45	27
	54	29
	48	26
	average = 49	average = 27.3

We have found that pWDH443 and pWDH444 is suitable for use according to the method of the invention, when light production (in response to DNA damage) is measured from cell extracts derived from cells according to the fourth aspect of the invention (in order that a good signal to noise ratio is achieved). pWDH443 and

pWDH444 are of limited use for whole cell measurements partly as a consequence of light scattering. There is only a narrow difference between the excitation (475 - 495nm) and emission (approximately 512nm) wavelengths used in GFP assessment. Using yEGFP-444, together with a sensitive spectrophotometer, it became possible to see the GFP signal as a correctly positioned shoulder on the fluorescence scan.

Figure 13 illustrates emission spectra (excitation at 488 nm) from 0.2 OD600 units of FF18984 cells untransformed and transformed cells with yEGFP-444 +/- 0.01% MMS. Cells were twice washed in sterile water before resuspending in 1x TE and further dilution. The lowest spectrum represents the fluorescence measured from untransformed cells with an MMS challenge and the next shows the same cells without MMS. The middle spectrum represents FF18984 cells transformed with the yEGFP-444 construct without an MMS challenge, whilst the upper spectrum shows the result of an MMS insult on the same transformed cells.

Other experiments were performed to refine the assay. To further reduce the impact of light scattering, a 495nm filter was introduced between the sample chamber and the emission-detector. This greatly enhanced the sensitivity of the instrument such that the difference between uninduced and induced cells was the same as that obtained with cell extracts. Furthermore when cells were grown in F1 medium (*supra*), it was not necessary to wash cell samples before transferring to the spectrophotometer.

We also performed experiments to demonstrate that the light output from cells according to the third aspect of the invention was specific to DNA damage and not a cell cycle phenotype. These experiments involved exposing transformed cells to hydroxyurea and nocodazole which can delay mitosis in different ways. Hydroxyurea interferes with DNA synthesis at two levels: it inhibits ribonucleotide reductase, so limiting the supply of precursors for DNA synthesis, and it inhibits DNA ligase, an enzyme responsible for repairing nicks (single strand breaks) in the DNA backbone.

In contrast nocodazole inhibits microtubule formation and thus primarily arrests cell growth by preventing the normal segregation of chromosomes during mitosis. At concentrations of these two chemicals that inhibited cell growth to a similar extent to our MMS treatment regime, there was no detectable induction of the GFP reporter gene which demonstrates that light emission was not related to the cell cycle and was induced by DNA damage inducing activation of the RAD54 promoter.

EXAMPLE 3

The modular design of pWDH443 (see Example 1) may also be exploited to allow simple replacement of the S65T derivative of GFP with the YEGFP gene to form preferred recombinant vector yEGFP-443 (see Fig. 11).

The method described in Example 2 may be followed to produce yEGFP-443 with the exception that pWDH443 was used instead of pWDH444.

Cells transformed with yEGFP-443 are also useful for use according to the method of the invention.

Cells transformed with yEGFP-444 and pWDH444 are particularly useful for detecting DNA damage without needing to disrupt cells to form an extract for assaying. The combination of the YEGFP gene, a sensitive host strain (e.g. FF18984), a low fluorescence medium (e.g. F1) and optimally the limitation of light scattering in the detecting device (e.g. using a 495nm filter) allows a shift away from the time consuming and complex assay of cell extracts to direct measurements of samples containing whole cells.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: University of Manchester Institute of Science and Technology
- (B) STREET: P.O. Box 88
- (C) CITY: Manchester
- (D) STATE: Greater Manchester
- (E) COUNTRY: United Kingdom
- (F) POSTAL CODE (ZIP): M60 1QD

(ii) TITLE OF INVENTION: DETECTION OF DNA DAMAGE

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1729 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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ACAACAAGGC AAATGTGGAT CTGTCATGTA TGGCAACGAC AGCAGGATGG CTCACAAAAA	300
AAGACAAAAA AACTAAGGC AAAAGAACAA AGCTCCTCTC CTGCTCAAGA AACGTATTGT	360
TGAAAAACCA CCGTCGTAAG AAAGTTTTTC TGTGACCTAT AATGGTTTAA AATCGGCCCA	420
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AGCGGAGAAG TGTGGCTAAA CCGCAAGTG CCTGCAAGAT CCACAGAACT AACCGCACGA	540
ACTGGCGGTC AGAAAAGAGC CTGTTCCGGA AAGAGAGAAA CAGAGAAACG ATCATGATGG	600

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CCTCCGGATC CGACATACGA TGACCTCAAT G

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCTCCGTTAA TTAACATCAG TTATAAGGAA ATATATATGG TACC

44

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5902 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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TTTCGTTTCAT CCATAGTTGC CTGACTCCCC GTCGTGTAGA TAACTACGAT ACGGGAGGGC 4680
TTACCATCTG GCCCCAGTGC TGCAATGATA CCGCGAGACC CACGCTCACC GGCTCCAGAT 4740
TTATCAGCAA TAAACCAGCC AGCCGGAAGG GCCGAGCGCA GAAGTGGTCC TGCAACTTTA 4800
TCCGCCTCCA TCCAGTCTAT TAATTGTTGC CGGGAAGCTA GAGTAAGTAG TTCGCCAGTT 4860
AATAGTTTGC GCAACGTTGT TGCCATTGCT ACAGGCATCG TGGTGTACG CTCGTCGTTT 4920
GGTATGGCTT CATTGAGCTC CGGTTCCCAA CGATCAAGGC GAGTTACATG ATCCCCCATG 4980
TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT CCTCCGATCG TTGTCAGAAG TAAGTTGGCC 5040
GCAGTGTTAT CACTCATGGT TATGGCAGCA CTGCATAATT CTCTTACTGT CATGCCATCC 5100
GTAAGATGCT TTTCTGTGAC TGGTGAGTAC TCAACCAAGT CATTCTGAGA ATAGTGTATG 5160
CGGCGACCGA GTTGCTCTTG CCCGGCGTCA ATACGGGATA ATACCGCGCC ACATAGCAGA 5220
ACTTTAAAG TGCTCATCAT TGGAAAACGT TCTTCGGGGC GAAAACCTCTC AAGGATCTTA 5280
CCGCTGTTGA GATCCAGTTC GATGTAACCC ACTCGTGAC CCAACTGATC TTCAGCATCT 5340
TTTACTTTCA CCAGCGTTTC TGGGTGAGCA AAAACAGGAA GGCAAAATGC CGCAAAAAAG 5400
GGAATAAGGG CGACACGGAA ATGTTGAATA CTCATACTCT TCCTTTTTC AATTATTGA 5460
AGCATTTATC AGGGTTATTG TCTCATGAGC GGATACATAT TTGAATGTAT TTAGAAAAAT 5520
AAACAAATAG GGGTCCGCG CACATTTCCC CGAAAAGTGC CACCTGACGT CTAAGAAACC 5580
ATTATTATCA TGACATTAAC CTATAAAAAT AGGCGTATCA CGAGGCCCTT TCGTCTCGCG 5640
CGTTTCGGTG ATGACGGTGA AAACCTCTGA CACATGCAGC TCCCGGAGAC GGTACAGCT 5700
TGTCTGTAAG CGGATGCCGG GAGCAGACAA GCCCGTCAGG GCGCGTCAGC GGGTGTGGC 5760
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ATACGATTTA GGTGACACTA TA 5902

```

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 909 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TTTAAATGC TTTCTGAAA CACGACTATT CTGATGGCTA ACGGTGAAAT TAAAGACATC	60
GCAAACGTCA CGGCTAACTC TTACGTTATG TGCGCAGATG GCTCCGCTGC CCGCGTCATA	120
AATGTCACAC AGGGCTATCA GAAAATCTAT AATATACAGC AAAAAACCAA ACACAGAGCT	180
TTTGAAGGTG AACCTGGTAG GTTAGATCCC AGGCGTAGAA CAGTTTATCA GCGTCTTGCA	240
TTACAATGTA CTGCAGGTCA TAAATTGTCA GTCAGGGTCC CTACCAAACC ACTGTTGGAA	300
AAAAGTGGTA GAAATGCCAC CAAATATAAA GTGAGATGGA GAAATCTGCA GCAATGTCAG	360
ACGCTTGATG GTAGGATAAT AATAATTCCA AAAAACCATC ATAAGACATT CCAATGACA	420
GTTGAAGGTG AGTTTGCCGC AAAACGCTTC ATAGAAGAAA TGGAGCGCTC TAAAGGAGAA	480
TATTTCAACT TTGACATTGA AGTTAGAGAT TTGGATTATC TTGATGCTCA ATTGAGAATT	540
TCTAGCTGCA TAAGATTGG TCCAGTACTC GCAGGAAATG GTGTTTTATC TAAATTCTC	600
ACTGGACGTA GTGACCTTGT AACTCCTGCT GTAAAAAGTA TGGCTTGGAT GCTTGGTCTG	660
TGGTTAGGTG ACAGTACAAC AAAAGAGCCA GAAATCTCAG TAGATAGCTT GGATCCTAAG	720
CTAATGGAGA GTTTAAGAGA AAATGCGAAA ATCTGGGGTC TCTACCTTAC GGTTTGTGAC	780
GATCACGTTT CGTACGTGC CAAACATGTA AGGCTTCATT ATGGAGATGG TCCAGATGAA	840
AACAGGAAGA CAAGGAATTT GAGGAAAAAT AATCCATTCT GGAAAGCTGT CACAATTTTA	900
AAGTTTAAA	909

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 756 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGTTAATTA ACTCTAAAGG TGAAGAATTA TTCCTGGTG TTGTCCCAAT TTTGGTTGAA	60
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ACTTACGGTA AATTGACCTT AAAATTATT TGTACTACTG GTAAATTGCC AGTTCCATGG	180
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AACCATTACT TATCCACTCA ATCTGCCTTA TCCAAAGATC CAAACGAAAA GAGAGACCAC	660
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AAATAACTGC AGGGCGCGCC ACTTCTAAAT AAGCGA	756

CLAIMS

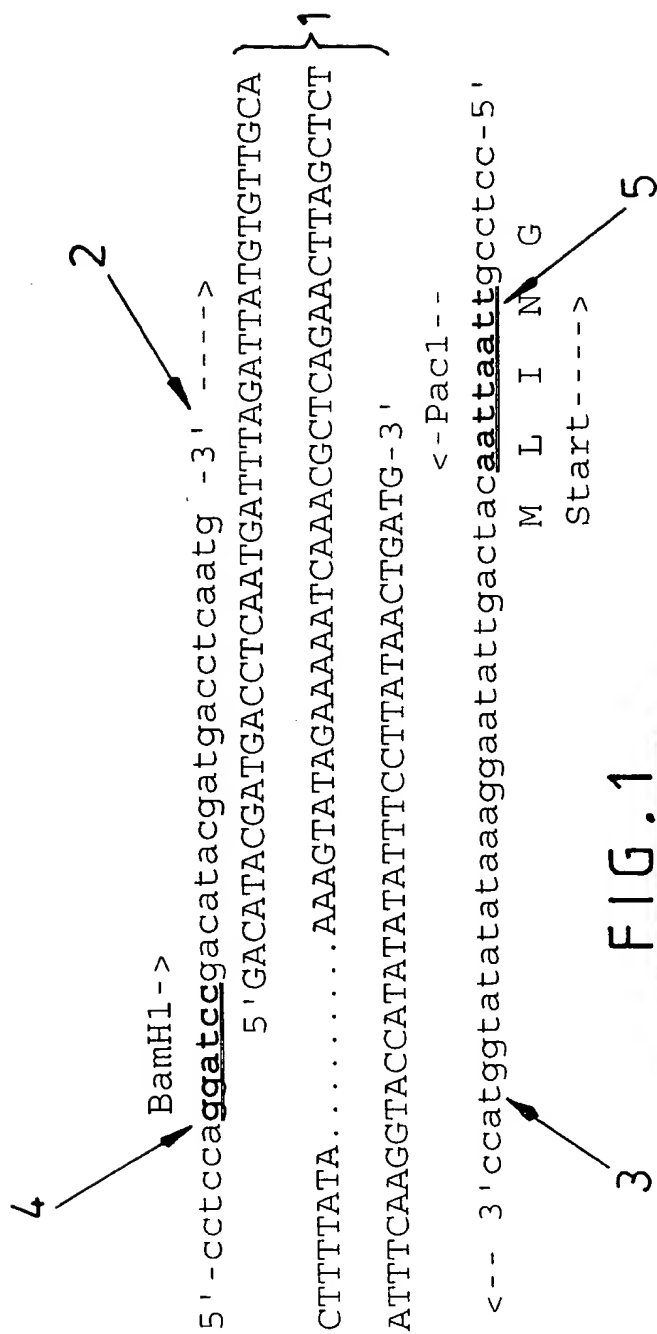
1. A recombinant DNA molecule comprising a regulatory element that activates gene expression in response to DNA damage operatively linked to a DNA sequence that encodes a light emitting reporter protein.
2. The recombinant DNA molecule according to claim 1, wherein the regulatory element contains a promoter sequence which induces DNA transcription.
3. The recombinant DNA molecule according to claims 1 or 2, wherein the regulatory element is associated with the regulation of the expression of DNA repair proteins.
4. The recombinant DNA molecule according to claim 3, wherein the regulatory element comprises the promoter and 5' regulatory sequences of the RAD54 repair gene.
5. The recombinant DNA molecule according to claim 4, wherein the regulatory element comprises the DNA sequence identified as SEQ ID NO 1 or a functional analogue or fragment thereof.
6. The recombinant DNA molecule according to claim 3, wherein the regulatory element comprises the promoter and 5' regulatory sequences of the RNR2 gene.
7. The recombinant DNA molecule according to any preceding claim, wherein the DNA sequence that encodes a light emitting reporter protein codes for Green Fluorescent Protein and light emitting derivatives thereof.

8. The recombinant DNA molecule according to claim 7, wherein the DNA sequence that encodes a light emitting reporter protein codes the S65T derivative of Green Fluorescent Protein.
9. The recombinant DNA molecule according to claim 7, wherein the DNA sequence that encodes a light emitting reporter protein codes the Yeast Enhanced derivative of Green Fluorescent Protein.
10. A recombinant vector comprising a DNA molecule according to any preceding claim and a DNA vector.
11. The recombinant vector according to claim 10, wherein the vector is designed to autonomously replicate in the cytosol of a cell.
12. The recombinant vector according to claim 11, wherein the vector contains DNA derived from the 2 μ plasmid.
13. The recombinant vector according to claim 10, wherein the vector is designed to integrate into the genome of a cell.
14. The recombinant vector according to claim 13, wherein the vector contains DNA encoding for part of the HO gene from chromosome IV of yeast.
15. The recombinant vector according to any one of claims 10 - 14, wherein the vector is derived from PFA vectors and derivatives thereof.
16. A recombinant vector PFA KANMX3GFP-RAD54 comprising the vector of sequence listing SEQ ID NO 4 and the regulatory element of sequence listing SEQ ID NO 1 inserted between the PacI and BamHI restriction enzyme sites of the vector of sequence listing SEQ ID NO 4.

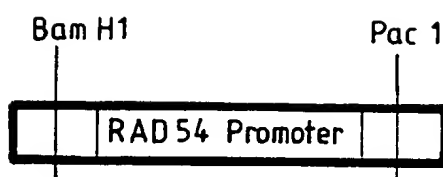
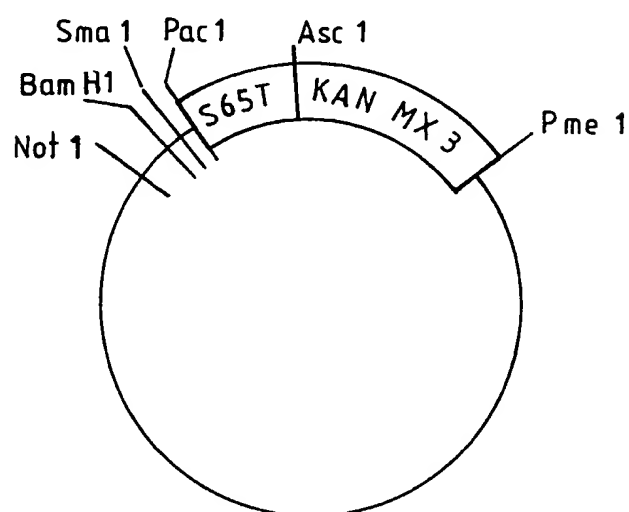
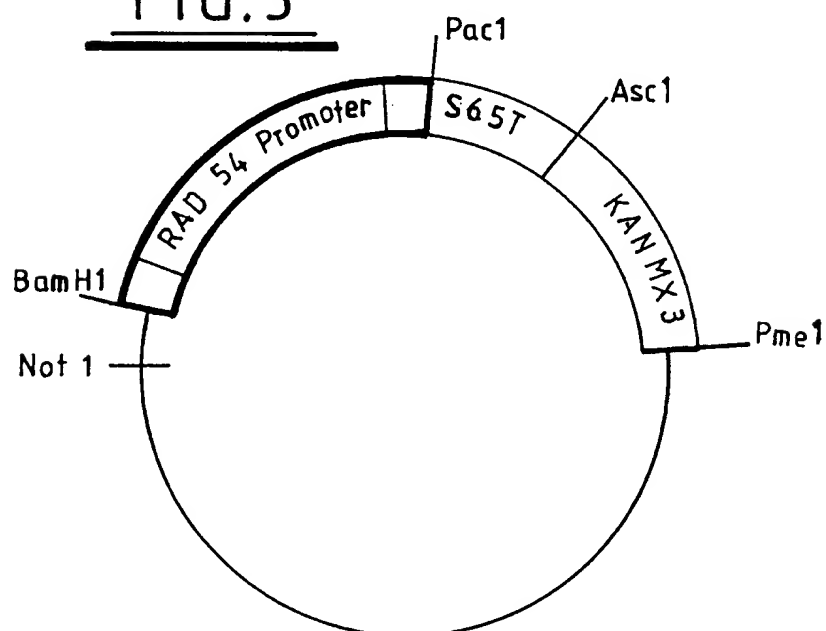
17. A recombinant vector pWDH443 comprising the recombinant vector according to claim 16 with a fragment of the HO gene corresponding to SEQ ID NO 5 inserted at the BamHI site of the vector.
18. A recombinant vector yEGFP-443 comprising the large fragment of the recombinant vector according to claim 17 generated by PacI and AscI digestion ligated to the large fragment of the YEGFP of SEQ ID NO 6 generated by PacI and AscI digestion.
19. A recombinant vector comprising a fragment of the 2 μ plasmid ligated with the large fragment generated from the BamHI and PmeI digestion of the recombinant vector according to claim 17.
20. A recombinant vector pWDH444 comprising the large HindIII/BamHI fragment released from the plasmid pRDK249 ligated with the large fragment generated from the BamHI and PmeI digestion of the recombinant vector according to claim 17.
21. A recombinant vector yEGFP-444 comprising the large fragment of the recombinant vector according to claim 20 generated by PacI and AscI digestion ligated to the large fragment of the YEGFP of SEQ ID NO 6 generated by PacI and AscI digestion.
22. A cell containing a DNA molecule according to any one of claims 1 - 9.
23. A cell containing a recombinant vector according to any one of claims 10 - 21.
24. The cell according to claims 22 or 23 wherein the cell is a unicellular organism.

25. The cell according to claim 24 wherein the cell is a yeast.
26. The yeast according to claim 25 which is Y486 in haploid form.
27. The yeast according to claim 25 named FF18984 or Y486 in haploid form..
28. The yeast according to claim 25 which is Y485/486 in diploid form.
29. A method of detecting for the presence of an agent that causes or potentiates DNA damage comprising subjecting a cell according to any one of claims 22 - 28 to an agent and monitoring the expression of the light emitting reporter protein from the cell.
30. The method according to claim 29, wherein agents are screened to assess whether it is safe to expose a living organism to such compounds.
31. The method according to claim 28 or 29, wherein the agents are candidate medicaments, food additives or cosmetics,
32. The method according to claim 28 or 29, wherein the agent is a contaminant of water supplies.
33. The method according to claim 28 or 29, wherein the agent is a contaminant of industrial effluents.
34. The method according to any one of claims 28 - 33, wherein the expression of light is measured from whole cells.

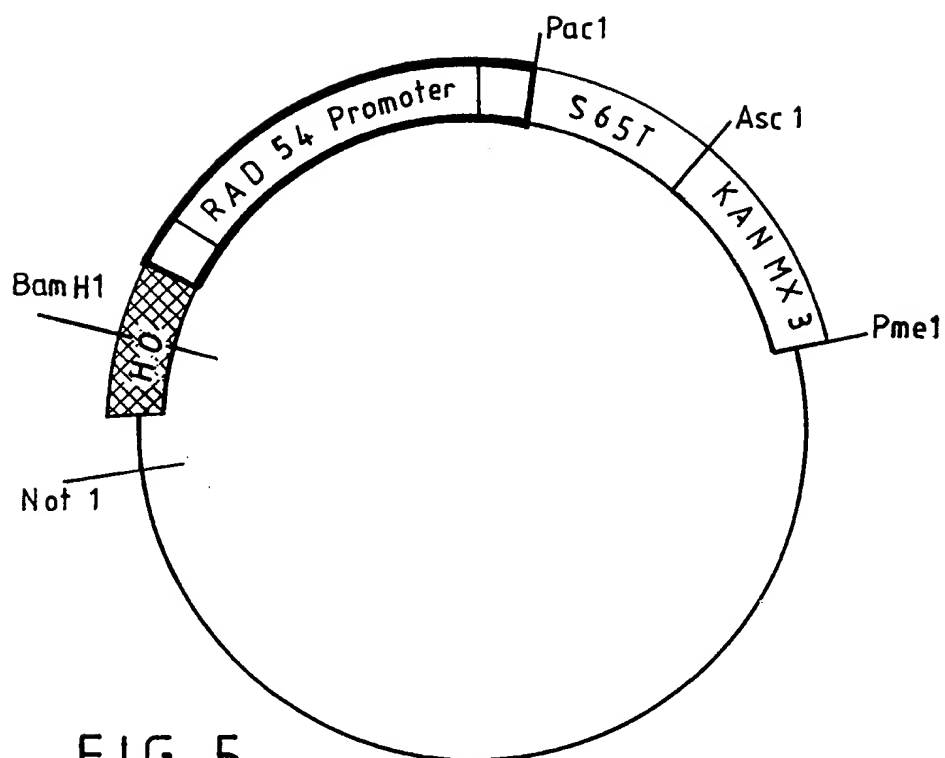
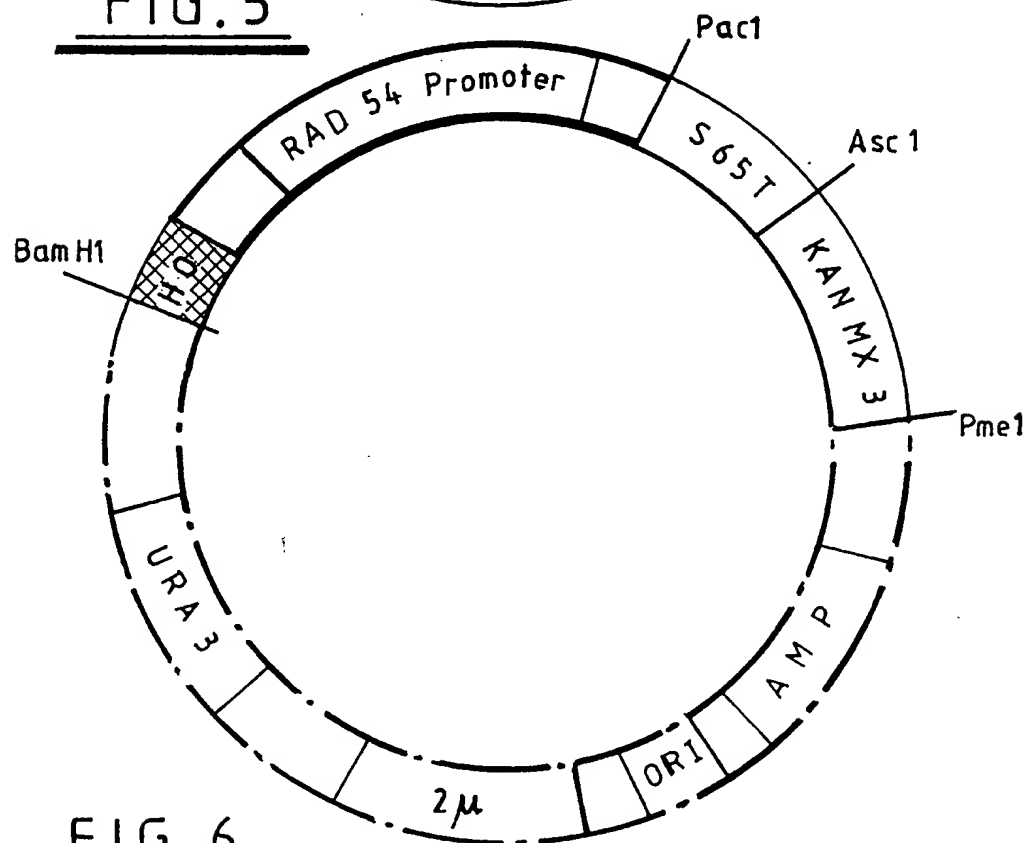
35. The method according to claim 34 comprising growing cells transformed with a recombinant vector according to claim 18 or 21, incubating the cells with the agent for a predetermined time and monitoring the expression of the light emitting reporter protein directly from a sample of the cells.
36. The method according to claim 35 wherein the cells are grown in a low fluorescence growth medium.
37. The method according to claim 36 wherein the low fluorescence growth medium is F1 medium.
38. The method according to any one of claims 28 - 33, wherein the expression of light is measured from an extract of the cells.

FIG. 1

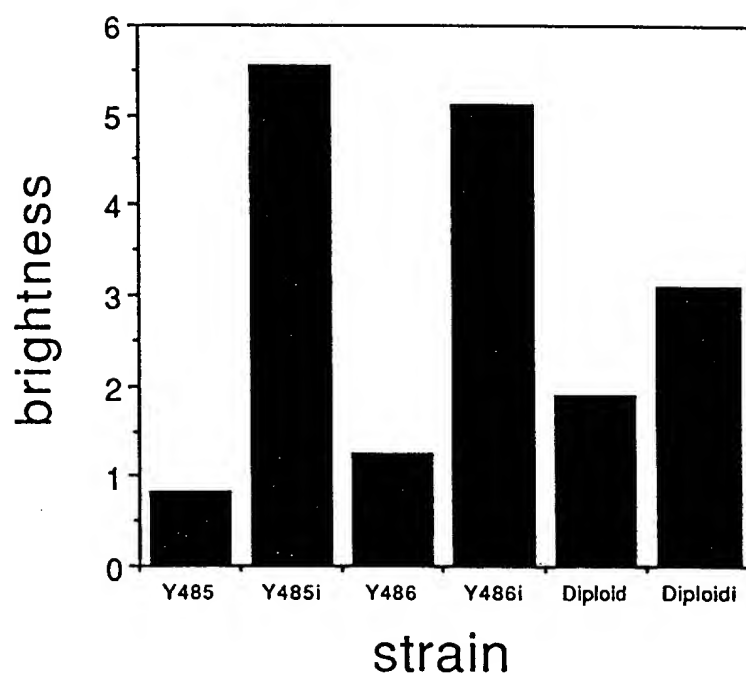
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FIG. 2FIG. 3FIG. 4

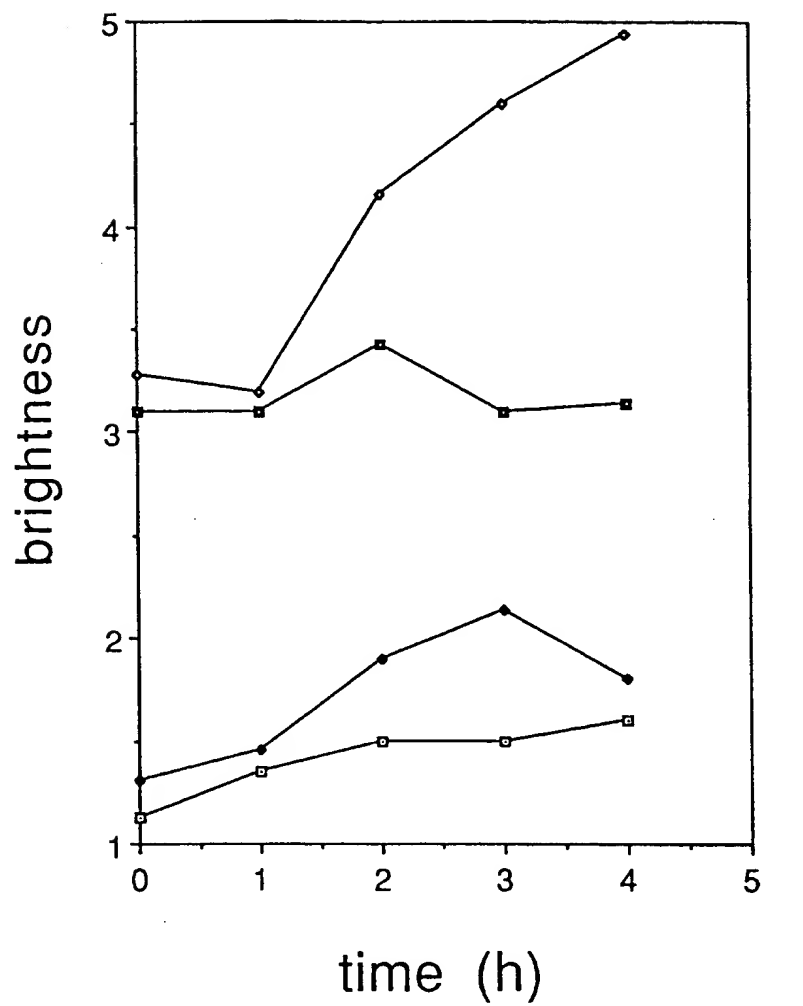
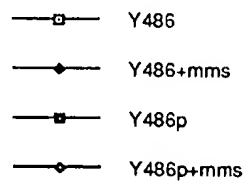
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FIG. 5FIG. 6

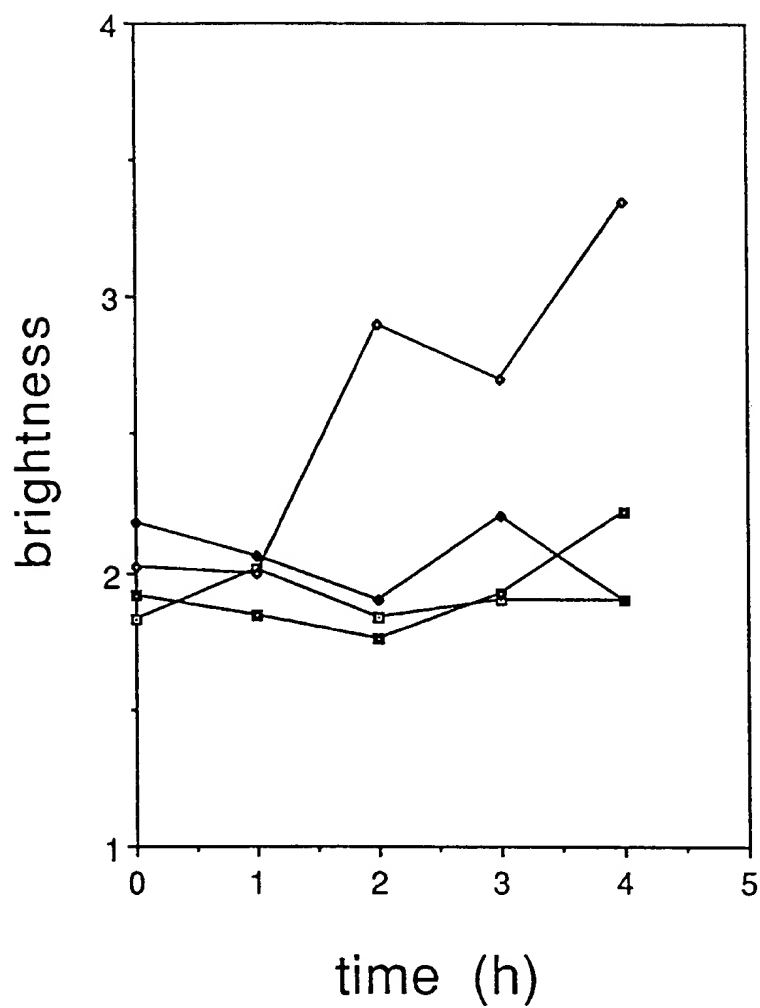
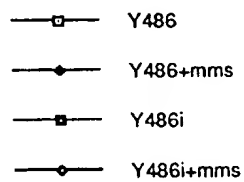
4/9

FIG. 7

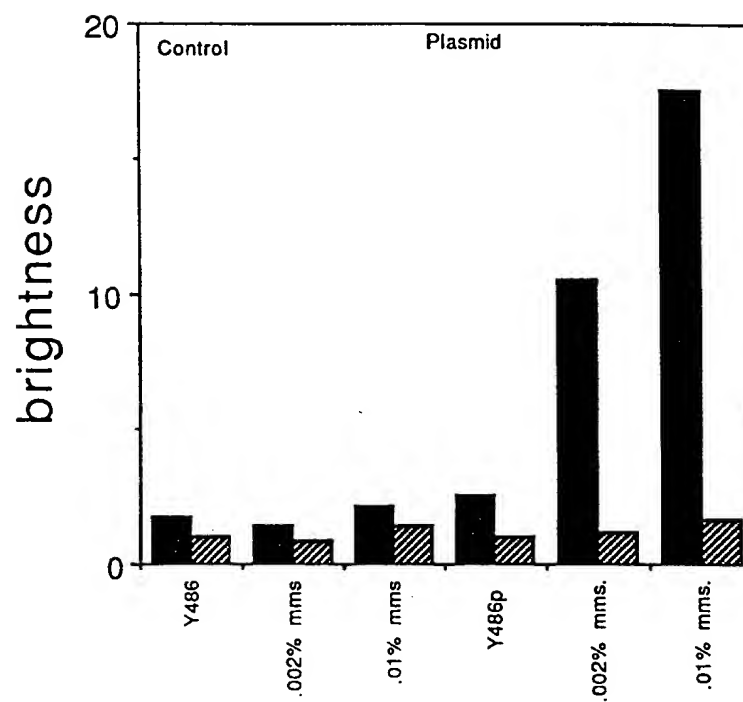
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FIG. 8

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FIG. 9

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FIG. 10

■ brightness
▨ bright SDS

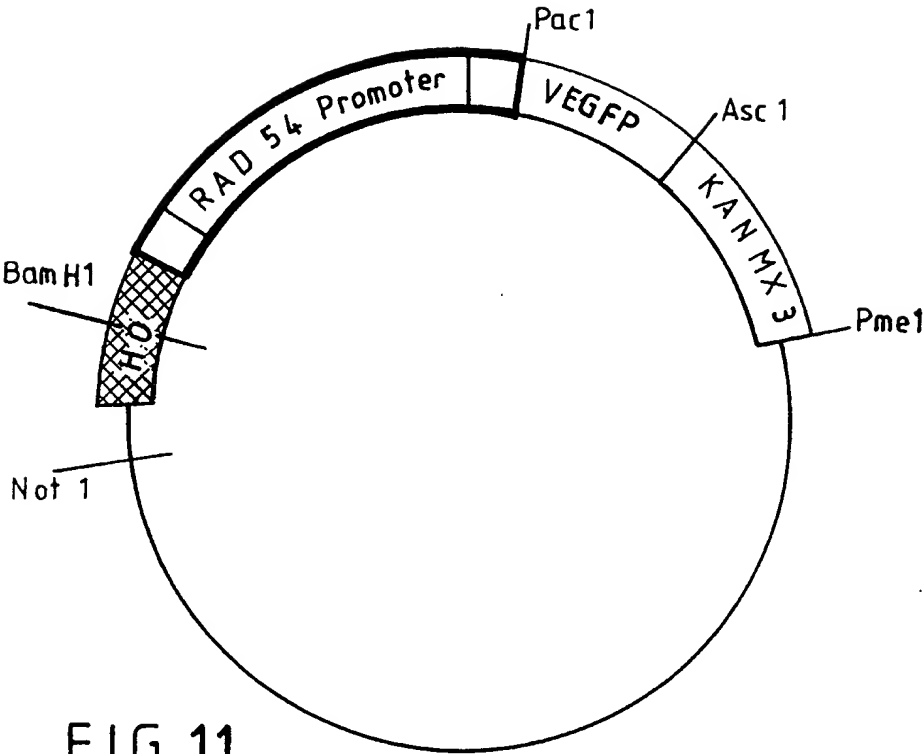


FIG.11

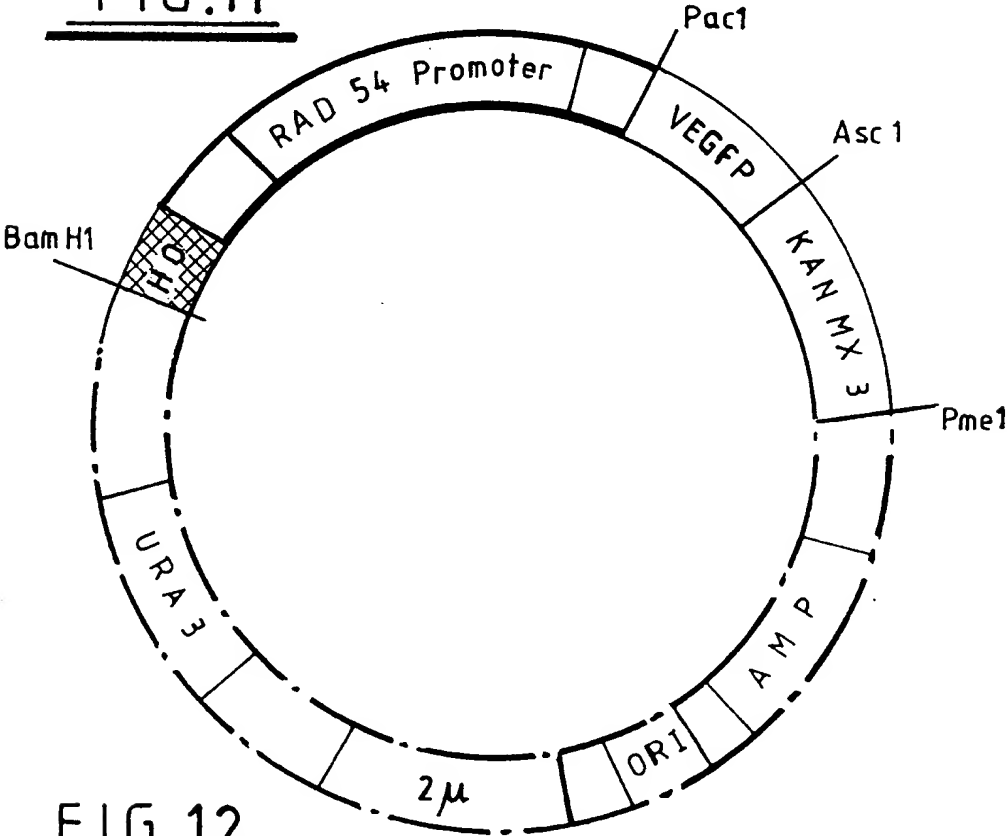
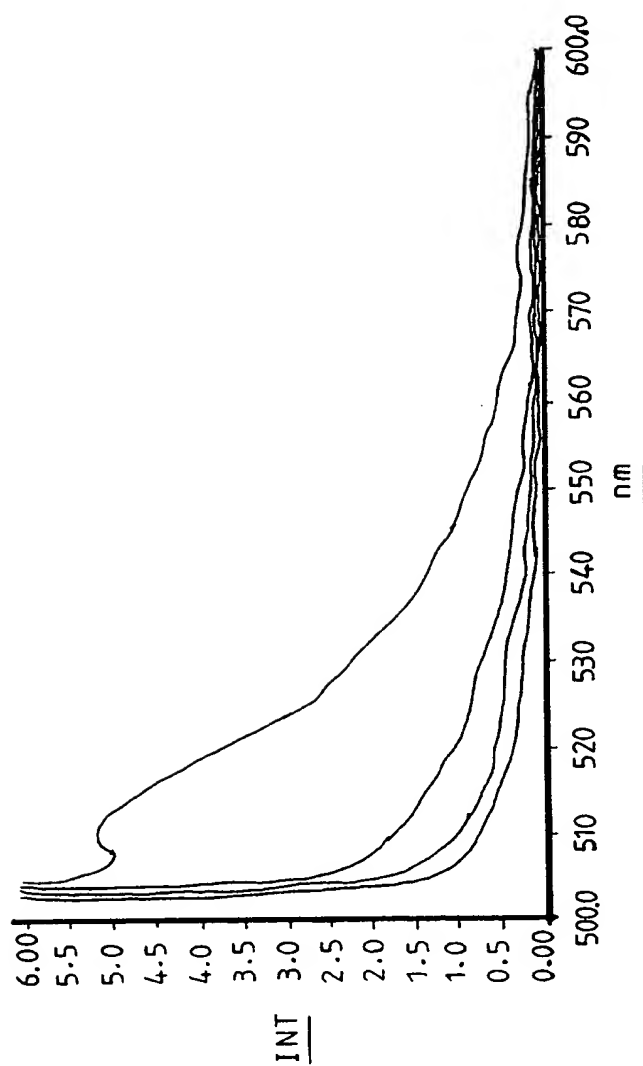


FIG.12

FIG. 13

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/00786

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/68 C12N15/81 C07K14/435 C07K14/395 C12N5/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 07463 A (UNIV COLUMBIA ;WOODS HOLE OCEANOGRAPHIC INST (US); CHALFIE MARTIN) 16 March 1995 cited in the application see page 7, line 5 - line 11 see page 16, line 16 - line 26 ----	1-38
Y	WO 94 17208 A (XENOMETRIX INC ;FARR SPENCER B (US); MARQUE TODD D (US)) 4 August 1994 see the whole document, especially page 28, lines 17-35 and page 34, lines 12-19 ----- -/--	1-38



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

3 August 1998

Date of mailing of the international search report

25/08/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Mandl, B

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/00786

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JAMES C. M. ET AL.: "DNA sequence analysis of a 35 kb segment from <i>saccharomyces cerevisiae</i> chromosome VII reveals 19 open reading frames including RAD54, ACE1/CUP2, PMR1, RCK1, AMS1 and CAL1/CDC43." YEAST, vol. 11, 1995, pages 1413-1419, XP002073528 see the whole document ---	4,5,16
Y	PLAUTZ J. D. ET AL.: "Green fluorescent protein and its derivatives as versatile markers for gene expression in living <i>Drosophila melanogaster</i> , plant and mammalian cells." GENE, vol. 173, 1996, pages 83-87, XP002073529 see page 85, right-hand column, last paragraph - page 86, left-hand column, line 2 ---	8
Y	CORMACK B. P. ET AL.: "Yeast-enhanced green fluorescent protein (yEGFP): a reporter of gene expression in <i>Candida albicans</i> ." MICROBIOLOGY, vol. 143, February 1997, pages 303-311, XP002073530 cited in the application see the whole document ---	9,18,21
Y	EP 0 163 491 A (BIOTECHNICA INTERNATIONAL) 4 December 1985 see the whole document, especially page 7, lines 13-18, page 14, 2nd paragraph and Figures ---	14,17-21
Y	WACH A. ET AL.: "New heterologous modules for classical or PCR-based gene disruptions in <i>Saccharomyces cerevisiae</i> ." YEAST, vol. 10, 1994, pages 1793-1808, XP002073531 cited in the application see the whole document, especially Figures ---	15-21
	-/--	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/00786

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>ELLEDGE S. J. AND DAVIS R. W.: "Identification of the DNA damage-responsive element of RNR2 and evidence that four distinct cellular factors bind it." MOLECULAR AND CELLULAR BIOLOGY, vol. 9, no. 12, 1989, pages 5373-5386, XP002073532 see the whole document, especially Figures and Discussion</p>	6
A	<p>COLE G. M. ET AL.: "Failure to induce a DNA repair gene, RAD54, in <i>Saccharomyces cerevisiae</i> does not affect DNA repair or recombination phenotypes." MOLECULAR AND CELLULAR BIOLOGY, vol. 9, no. 8, 1989, pages 3314-3322, XP002073533 see abstract</p>	4,5,16
A	<p>RUSSEL D. W. ET AL.: "Structure of the <i>Saccharomyces cerevisiae</i> HO gene and analysis of its upstream regulatory region." MOLECULAR AND CELLULAR BIOLOGY, vol. 6, no. 12, 1986, pages 4281-4294, XP002073534 see Fig.3 on page 4284</p>	14,17,20
A	<p>WELCH W.J.: "How cells respond to stress." SCIENTIFIC AMERICAN, vol. 5, May 1993, pages 34-41, XP002073535 see page 41, left-hand column, last paragraph - middle column, last paragraph</p>	1-38
P,X	<p>WALMSLEY R. M. ET AL.: "Green fluorescent protein as a reporter for the DNA damage-induced gene RAD54 in <i>Saccharomyces cerevisiae</i>." YEAST, vol. 13, December 1997, pages 1535-1545, XP002073536 see the whole document</p>	1-5,7,8, 10,17, 19,20, 22-25,27

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 98/00786

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9507463 A	16-03-1995	US 5491084 A AU 7795794 A CA 2169298 A EP 0759170 A JP 9505981 T	13-02-1996 27-03-1995 16-03-1995 26-02-1997 17-06-1997
WO 9417208 A	04-08-1994	AT 160178 T AU 692434 B AU 6124394 A DE 69406772 D DE 69406772 T EP 0680517 A ES 2111289 T JP 9503121 T	15-11-1997 11-06-1998 15-08-1994 18-12-1997 12-03-1998 08-11-1995 01-03-1998 31-03-1997
EP 0163491 A	04-12-1985	AT 136059 T AU 4270985 A BR 8502400 A CA 1338857 A DE 3588096 D DE 3588096 T DK 224185 A EP 0707068 A FI 852024 A JP 61040793 A US 5422267 A	15-04-1996 28-11-1985 21-01-1986 21-01-1997 02-05-1996 05-09-1996 23-11-1985 17-04-1996 23-11-1985 27-02-1986 06-06-1995